(b) *Developing solvent.* Mix ethyl acetate, pyridine, *n*-butanol, acetic acid, and water in volumetric proportions of 42:21:21:6:10, respectively.

(c) *Spray solution.* Immediately before use, mix 100 milliliters of a 1-percent ferric chloride solution in 1 percent hydrochloric acid with 100 milliliters of a 1-percent potassium ferricyanide solution and 75 milliliters of methanol.

(d) *Preparation of working standard solution.* Prepare a solution containing approximately 2.5 milligrams per milliliter of cefoxitin working standard in distilled water.

(e) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate for 1 hour. Prepare a plate as follows: On a line 2 centimeters from the base of the silica gel plate, and at intervals of 2 centimeters, spot 10 microliters each of the standard solution and the sample solution. After all spots are thoroughly dry, place the silica gel plate directly into the glass trough. Cover and seal the tank. Allow the solvent front to travel about 15 centimeters from the starting line. Remove the plate from the tank and heat it for 1 hour at 60° C in a circulating air oven. Remove the plate from the oven and allow it to cool at room temperature. Apply the spray solution and allow it to air dry. After approximately 15 minutes, the compound appears as a blue spot on a yellow-green background.

(f) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The sample and standard should have spots of corresponding R_f values.

[44 FR 10373, Feb. 20, 1979, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.327 Thin layer chromatographic identity test for cyclacillin.

(a) *Equipment*—(1) *Chromatography tank.* Use a rectangular tank approximately 23 x 23 x 9 centimeters, with a glass solvent trough on the bottom and a tight-fitting cover.

(2) *Plates.* Use 20 x 20 centimeter thin layer chromatography plates coated with Silica Gel G or equivalent to a thickness of 250 microns.

(b) *Reagents*—(1) *Developing solvent.* One percent ammonium formate aqueous solution.

(2) *Spray solution.* Dilute starch iodide paste TS (U.S.P. XIX) with an equal volume of water. Mix diluted starch iodide paste, glacial acetic acid, and 0.1*N* iodine in volumetric proportions of 50:3:1, respectively.

(c) *Assay solutions*—(1) *Preparation of working standard solution.* Accurately weigh an amount of cyclacillin working standard and dissolve the material with sufficient 0.1*N* sodium hydroxide to obtain a solution containing 1 milligram per milliliter. Allow the solution to stand for 15 minutes before using.

(2) *Preparation of sample solution.* Using the sample solution prepared as described in the section for the antibiotic to be tested, proceed as described in paragraphs (d) and (e) of this section.

(d) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank. Cover and seal the tank. Allow it to equilibrate. Prepare a plate as follows: On a line 2 centimeters from the base of the thin layer chromatography plate and at intervals of 2 centimeters, spot 5 microliters each of the working standard solution and sample solution. Dry the spots thoroughly with a stream of dry air. Place the plate in the trough in the chromatography tank. Cover and seal the tank. Allow the solvent front to travel about 15 centimeters from the starting line and then remove the plate from the tank. Dry the plate by heating for 30 minutes at 80° C in a circulating air oven. Visualize the spots by applying the spray solution.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line, and the distance the spots are from the starting line. Divide the latter by the former to calculate the R_f value. The sample and standard should appear as white spots against a blue background at an R_f of approximately 0.6. The test is satisfactory if

the R_f value of the sample compares with that of the working standard.

[46 FR 2981, Jan. 13, 1981, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.328 High pressure liquid chromatographic assay for sulfisoxazole acetyl content.

(a) *Equipment.* A suitable high pressure liquid chromatograph, such as a Waters Associates Model 244[1] or equivalent equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with a suitable reverse phase packing such as: Waters Associates, Micro-Bondapak C18;[1] and

(6) A suitable integrator.

(b) *Reagents*—(1) *Mobile phase.* Mix acetonitrile (high pressure liquid chromatography grade): water (40:60). Filter the mobile phase through a suitable glass fiber filter or equivalent which is capable of removing particulate contamination to 1 micron in diameter. De-gas the mobile phase just prior to its introduction into the chromatograph pumping system.

(2) *Internal standard solution.* Dissolve 0.33 milligram of benzanilide per milliliter in acetonitrile (high pressure liquid chromatography grade). Filter the solution through a suitable glass fiber filter or equivalent which is capable of removing particulate contamination to 1 micron in diameter.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.2 milliliters per minute. Use a detector sensitivity setting that gives a peak height for reference standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the baseline.

(d) *Preparation of the working standard and sample solutions*—(1) *Working standard solution.* Prepare a solution containing 1.0 milligram per milliliter of sulfisoxazole acetyl in the internal standard solution.

(2) *Sample solution.* Reconstitute the sample as directed in the labeling. Allow to stand for 1 hour. Shake gently and transfer 5.0 milliliters of the sample to a separatory funnel. Extract the suspension three times with 75-milliliter portions of chloroform. Collect the chloroform layers in a 250-milliliter volumetric flask. Dilute the flask to volume with chloroform and mix. Filter a portion of the solution through a suitable glass fiber filter or equivalent which is capable of removing particulate contamination to 1 micron in diameter. Transfer a 4.0-milliliter aliquot of the filtrate into a 25-milliliter glass-stoppered flask and evaporate to dryness under a stream of dry air. Dissolve the residue in 10.0 milliliters of the internal standard solution, stopper, and mix.

(e) *Procedure.* Using the equipment, reagents, and operating conditions listed in paragraphs (a), (b), and (c) of this section, inject 5 microliters of sample or working standard solution prepared as described in paragraph (d) of this section, into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components. The elution order is void volume, sulfisoxazole acetyl and benzanilide.

(f) *Calculations.* Calculate the sulfisoxazole content as follows:

$$\text{Milligrams of sulfisoxazole per milliliter of sample} = \frac{A \times \text{Concentration of the standard solution in milligrams per milliliter} \times 125 \times 0.864}{B}$$

[1] Available from: Waters Associates, Inc., Maple Street, Milford, MA 10757.